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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Vered HORNIK et al.

Application No.: 09/644,456

Filing Date: August 24, 2000

For: CONFORMATIONALLY CONSTRAINED BACKBONE CYCLIZED INTERLEUKIN-6 ANTAGONISTS

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Examiner: Michael Borin

Attorney Docket No.: 87534-3100

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Sir:

Applicants have claimed priority under 35 U.S.C. § 119 of Israeli Application No. 130238 filed June 1, 1999. In support of this claim, a certified copy of said application is submitted herewith.

No fee or certification is believed to be due for this submission. Should any fees be required, however, please charge such fees to Winston & Strawn LLP Deposit Account No. 50-1814.

Respectfully submitted,

7/01/04
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בקשה לפטנט
Application for Patent

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אני, (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)
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The Inventors:

בעל אמצאה מכח החוק The law שמה הוא
Owner, by virtue of of an invention the title of which is

(בעברית) **מעכבים מקובעי מבנה של אינטרלוקין-6 בעלי ציקליזציה שלד**
(Hebrew)

(באנגלית) CONFORMATIONALLY CONSTRAINED BACKBONE CYCLIZED INTERLEUKIN-6 ANTAGONISTS
(English)

מבקש בזאת כי ינתן לי עליה פטנט hereby apply for a patent to be granted to me in respect thereof.

*בקשת חלוקה - Application of Division	*בקשת פטנט מוסף - Application for Patent Addition	*דרישה דין קדימה Priority Claim		
מבקשת פטנט from Application	*לבקשה/לפטנט to Patent/Appl.	מספר/סימן Number/Mark	תאריך Date	מדינת האגוד Convention Country
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CONFORMATIONALLY CONSTRAINED BACKBONE CYCLIZED INTERLEUKIN-6 ANTAGONISTS

מעכבים מקובעי מבנה של אינטרלוקין-6 בעלי ציקליזציית שלד

P/015

CONFORMATIONALLY CONSTRAINED BACKBONE CYCLIZED INTERLEUKIN-6 ANTAGONISTS

FIELD OF THE INVENTION

The present invention relates to conformationally constrained backbone-cyclized IL-6 antagonists, and to pharmaceutical compositions containing same.

BACKGROUND OF THE INVENTION

Interleukin-6 (IL-6) is a member of the helical cytokine family. IL-6 is produced by almost all cell types in response to a variety of different stimuli including bacterial (LPS) and viral infections, cancer, and other cytokines (e.g. IL-1). IL-6 is a pleiotropic factor, it participates in numerous processes and is thus associated with numerous disorders.

Bioactivity of IL-6 requires interaction of the cytokine, IL-6, its receptor (IL-6R) and a transmembrane signal transducer known as glycoprotein 130 (gp130), and formation of a hexameric complex containing two units of each protein. The outcome of the complex formation is dimerization of gp130, which by itself is sufficient for obtaining IL-6 like bioactivity (Fourcin, et al., J. Biol. Chem. 271: 11756, 1996.). Several other cytokines also use gp130 for signal transduction. These include: interleukin-11 (IL-11), ciliary neurotrophic factor (CNTF), leukocyte inhibitory factor (LIF), oncostatin M (OSM).

IL-6 and host defense

IL-6 levels increases early during bacterial and viral infections. IL-6 induces production of acute phase proteins which are thought to participate in the defense of the host organism against tissue damage and infection. The acute phase response is considered to be the systemic inflammatory reaction to infection and injury.

IL-6 also amplifies the immune system through its multiple

growth and differentiation activities such as induction of B cell differentiation, replication of bone marrow progenitor cells, and augmentation of T lymphocytes, including enhancement of cytotoxic T lymphocytes.

IL-6 in homeostasis, injury and transplantation:

IL-6 levels are increased during stress. Increased bone resorption and increased osteoclast activity in postmenopausal osteoporosis have been linked with IL-6. Estrogen loss induced by ovariectomy in mice enhances osteoclast development and this change can be prevented by antibodies to IL-6.

IL-6 in rabbits is directly responsible for elevation of body temperature. High IL-6 levels in burn patients correlates with mortality. Elevated IL-6 levels are associated with traumatic events and allograft rejection.

IL-6 in immune disorders

Elevated levels of IL-6 in cardiac myxoma and cervical carcinoma are associated with autoimmunity indications such as: production of anti-nuclear factor, rheumatoid factors, elevated immune complexes, arthritis and nephritis.

Castelman's disease patients suffer form fever, anemia, hyper-γ-globulinemia, and an increase in acute phase proteins. The lymph nodes constitutively produce large amounts of IL-6, and surgical removal of the involved lymph nodes is followed by decrease in serum IL-6 levels and a dramatic clinical improvement. It has been demonstrated that systemic manifestation of Castelman's disease could be alleviated by treatment with anti-IL-6 antibody.

Local and general symptoms of rheumatoid arthritis, such as plasma cell infiltration into synovial tissues, autoantibody production, and polyclonal hyper-γ-globulinemia can be explained by increased IL-6 production observed in synovial tissue. At least two mediators which are elevated in RA

patients, PGE2 and IL-1, are known to induce synthesis of IL-6. Higher than normal levels of IL-6 have been detected in sera of patients with active SLE. Increased plasma levels of IL-6 were observed in psoriasis patients.

AIDS:

High levels of IL-6 are associated with HIV infection. The HIV envelope glycoproteins gp120 and gp160 induce IL-6 production from CD4+ T cells. Is has been demonstrated that IL-6 is a growth factor of the AIDS associated Kaposi's sarcoma (Murakamin-Mori, et al. Int. Immunol. 8: 595, 1996). The soluble form of the IL-6 receptor (sIL-6Ra) is a potent growth factor for AIDS-associated Kaposi's sarcoma (KS) cells. The soluble form of gp130 is antagonistic for sIL-6Ra-induced AIDS-KS cell growth. Furthermore, high IL-6 levels are associated with weight loss in AIDS.

Proliferative diseases and malignancies

An autocrine role for IL-6 has been reported in several types of cancer, among which are renal cell carcinoma, Hodgkin and non-Hodgkin's lymphoma, chronic lymphocytic leukemia, and acute myeloid leukemias.

Plasmacytoma and myeloma cells require IL-6 for growth. Treatment of primary plasma cell leukemia with anti-IL-6 antibodies improves the patient's clinical status throughout the treatment. Also, IL-6 deficient mice are completely resistant to plasmacytoma induction.

Multiple myeloma is a malignant proliferation of plasma cells derived from a single clone. It is manifested in a number of organ dysfunctions and symptoms of bone pain or fractures, hypercalcemia, renal failure, susceptibility to infection, anemia and bleeding. The disease typically follows a chronic course for 2 to 5 years before progressing into an acute terminal phase.

The different therapeutic strategies for inhibition of multiple myeloma have been recently reviewed (Ogata A., Leuk. Res. 20 :

303, 1996). The vast majority of multiple myeloma patients require systemic chemotherapy to control the malignancy, and symptomatic supportive care to minimize the morbidity. The outcome of patients with multiple myeloma is still unsatisfactory, with median survival times of 2 to 3 years. Clearly, there is a need for an agent that cannot only improve the chances of remission, but also increase the duration of response and enhance survival.

Recently, injection of an anti-IL-6 antibody was tested in young population and resulted in a complete blocking of myeloma cell proliferation and inhibition of the serum IL-6 bioactivity. However, the administration of a single anti-IL-6 mAb appeared to be insufficient (Klein, et al., Blood 85: 863, 1995).

Inhibitors of IL-6 activity

It is generally accepted that IL-6 inhibitors have clinical value. As indicated above there are a number of clinical situations where IL-6 inhibitors could be of therapeutic use. Most of the attempts to produced inhibitors to IL-6 reported in the literature in the past, used proteins. In general, proteins are not very suitable as drugs, due to their immunogenic potential, high cost, and the necessity for parenteral administration. The various attempts to use proteins to inhibit IL-6 are described below.

Monoclonal antibodies and antibody fragments:

The most common approach is to use monoclonal antibodies (mAbs). Several murine mAbs capable of inhibiting the bioactivity of IL-6 have been described.

The drawbacks in the use of antibodies against IL-6 are that the mAb traps the IL-6 in an immune complex in the circulation (May et al., J. Immunol. 151, 3225, 1993), thereby increasing its half-life 200-fold (Lu et al., Blood 86, 3123,1995). The immune complexes are thus serve as long term, slow release deposits of IL-6. The presence of high levels of circulating immune complexes could result in their precipitation in the

basal lamina in the kidneys or in the joints, which could lead to kidney failure or arthritis.

Attempts to block IL-6 with monoclonal antibodies have been reported for the following diseases: AIDS associated syndromes and lymphoma (Emilie, et al., Blood 84: 2472, 1994), Castelman's disease, rheumatoid arthritis (Wijdenes et al., J. Interferon Res. 14: 297, 1994), multiple myeloma, plasma cell leukemia (Klein et al., Blood 78: 1198, 1991), and endotoxin toxicity. Partial response were observe in most instances, but the problems associated with the use of monoclonal antibodies for inhibition of IL-6 have so far prevented their routine clinical application.

Some of problems associated with the clinical use of monoclonal antibodies are a result of the large size of the antibody molecule. Minibodies which utilize the hypervariable loop structure of antibodies, capable of inhibiting IL-6 bioactivity were recently reported (Martin et al., The EMBO J. 13, 5303, 1994). Binding of IL-6 to a minibody molecule should create a complex that is small enough to be secreted from the kidney, thereby decreasing the risk of creating slow release IL-6 deposits. Minibody-IL-6 complexes may not be recognized as immune complexes, thereby decreasing the chances for kidneys and arthritic problems. The minibodies could still be immunogenic and it is unlikely that they will be orally available. So far, minibodies with sufficiently high affinity for IL-6 have not been obtained.

Mutated proteins:

Several IL-6 mutants were selected for desired activity using phage display systems. Super active mutants were reported (Toniatti, et al., The EMBO J. 15: 2726, 1996) as well as mutants which retain the capacity for binding of the IL-6R but lose the ability for interaction with the gp130 and thus could serve as functional antagonists of IL-6 bioactivity (Savino et al., The EMBO J. 13,5863, 1994; Sporeno, et al., Blood 87: 4510, 1996). The danger in clinical use of such mutants is the

formation of antibodies that would recognize both the mutated and the native molecules. Such antibodies could block the bioactivity of IL-6 long after the treatment is terminated thereby exposing the patients to danger associated with lack of IL-6.

US Patent No. 5,470,952 disclose CTNF and IL-6 antagonists which are heterodimer proteins comprising a soluble α specificity determining cytokine receptor component and the extracellular domain of a β receptor component. Specifically, the inventors claim an IL-6 antagonist, capable of binding IL-6 to form a nonfunctional complex, comprising: soluble IL-6Rα and the extracellular domain of gp130.

Cytokine-toxin conjugates:

Several applications of IL-6 inhibitors entail the elimination of IL-6 dependent tumors, such as multiple myeloma. This goal can be achieved by the use of IL-6-toxin conjugate (Jean and Murphy, Prot. Eng. 4, 989, 1991). Malignant cells that have receptor for IL-6 would bind the toxin via the IL-6 portion of the conjugate and would be eliminated by toxin activity. Toxicity to all non-malignant cells that also express the IL-6 receptor is a dangerous possibility. Since IL-6 is required for development of normal humoral and cellular immune response, it is possible to speculate that treated patients would immunocompromised.

Peptide antagonists of IL-6

Grube and Cochran identified a regulatory domain of the IL-6 receptor (J. Biol. Chem. 269: 20791, 1994). The region is from the extramembranal domain of the IL-6R and it is involved in the regulation of IL-6 signal transmission. A synthetic peptide, corresponding to residues 249-264 of the IL-6R inhibits IL-6-dependent cell mitogenesis and IL-6-stimulated acute phase response without affecting ligand binding.

In a search for possible lead compounds, epitope mapping of the human IL-6R was carried out (Halimi et al., Eur. Cytokin, Netw. 6:135, 1995) with mAbs to IL-6R which inhibit the biological activity of IL-6 (Novick, et al., Hybridoma 10, 137, 1991). The 10 mer linear peptides that were recognized by two of the antibodies are from the same region identified by Grube and Cochran (ibid). The peptides identified by these two groups are indicated in the frame of the IL-6R sequence in figure 1.

International PCT application WO 97/13781 discloses these synthetic peptides and analogs derived from the IL-6 that inhibit IL-6 activity. The peptides claimed are characterized also by being a linear epitope recognized by one or more Mabs specific to IL-6R. Peptides 1122 and 1123 (Halimi et al. ibid), were synthesized and found to inhibit IL-6 bioactivity in vitro with an ID_{50} of about 100 μM.

International PCT application WO 97/48728, discloses synthetic peptides which derived from IL-6 and from IL-6 receptor (either the IL-6R or gp130), and have IL-6 antagonistic or agonistic activity. The peptides interact with the receptor site of IL-6 or with IL-6Rs present at target cells or when combined, interact with both sites (IL-6 and IL-6R).
US Patent No. 5,210,075 discloses IL-6 antagonist peptides of varying length, which are modeled after a portion of the sequence of IL-6 itself (p51-70 Hirano et al. Nature 324:73, 1986), or which are modeled after four different portions of the sequence of the IL-6 receptor molecule.

None of these disclose conformationally constrained IL-6 peptide antagonists which are cyclized as described in the present invention.

Peptide mimetic and backbone cyclized peptide analog antagonists of IL-6

It is most beneficial to produce conformationally constrained

peptide analogs overcoming the drawbacks of the native peptide molecules (low metabolic stability, poor oral bio-availability, rapid liver and kidney excretion, and lack of selectivity), thereby providing improved therapeutic properties.

A novel conceptual approach to the conformational constraint of peptides was introduced by Gilon, et al., (Bio-polymers 31:745, 1991), who proposed backbone to backbone cyclization of peptides. The theoretical advantages of this strategy include the ability to effect cyclization via the carbons or nitrogens of the peptide backbone without interfering with side chains that may be crucial for interaction with the specific receptor of a given peptide.

Further disclosure by Gilon and coworkers (WO 95/33765) provided methods for producing building units required in the synthesis of backbone cyclized peptide analogs. Recently, The successful use of these methods to produce backbone cyclized peptide analogs having somatostatin activity was also disclosed (WO 98/04583).
Libraries of backbone cyclized analogs including IL-6 analogs are disclosed in international application WO 97/09344. In that disclosure, a selection method termed "Cycloscan", based on conformationally constrained backbone cyclic peptide libraries that allows rapid detection of active analogs derived from a given sequence is described.

Nowhere in the background art, are backbone cyclized peptide analogs shown to possess IL-6 inhibitory activity.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide novel peptide analogs, which are characterized in that they incorporate building units with bridging groups attached to the

alpha nitrogens of alpha amino acids, having IL-6 inhibitory activity. Specifically, these compounds are backbone cyclized IL-6 antagonists comprising a peptide sequence of five to twenty amino acids that incorporates at least one building unit, said building unit containing one nitrogen atom of the peptide backbone connected to a bridging group comprising an amide, thioether, thioester or disulfide, wherein the at least one building unit is connected via said bridging group to form a cyclic structure with a moiety selected from the group consisting of a second building unit, the side chain of an amino acid residue of the sequence or the N-terminal amino acid residue. Preferably, the peptide sequence incorporates six to twelve amino acids, having IL-6 inhibitory activity.

Bioactivity of IL-6 requires each of the molecules in the tripartite complex, i.e. IL-6, IL-6R and gp130 signal transducer, to interact with the two other partners. The objectives of the present invention will be achieved by peptides that inhibit any one of interactions in the complex as follows:

a) Peptides derived from IL-6 that interfere with IL-6-IL-6R interaction or with IL-6/gp130 interaction.

b) Peptides derived from IL-6R that interfere with IL-6-IL-6R interaction or with IL-6R/gp130 interaction.

c) Peptides derived from gp130 that interfere with IL-6/gp130 or with IL-6R/gp130 interactions.

According to one aspect of the present invention, the segment of the IL-6R spanning residues 247-271 is currently a most preferred embodiment for development of conformationally constrained backbone cyclized peptide analogs to be used as an inhibitor of IL-6 activity.

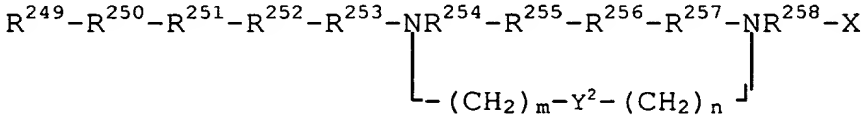
From the massive screening of backbone cyclized peptide analogs, three preferred peptide analogs were unexpectedly found to have significantly enhanced inhibitory activity in comparison to the linear epitope. These backbone cyclized

peptide analogs mimic the IL-6R inhibitory domain of residues 249-258. But unlike the previously described peptides derived from this domain, these novel backbone cyclized peptide analogs possess unique features which make them more suitable for use in pharmaceutical compositions for treatment of pathological conditions associated with elevated levels of IL-6.

According to the present invention it is now disclosed that more preferred backbone cyclized analogs are decapeptide and nonapeptide antagonists of IL-6 with improved activity and metabolic stability. Additional more preferred analogs may advantageously include at least one D-isomer of amino acids in their sequence.

Preferably, the backbone cyclized analogs of the present invention are derived from, or mimic the sequence of the IL-6R molecule, preferably related to residues 247-271 of the IL-6R amino acids sequence. Additional preferred analogs are derived from the sequence of the IL-6 molecule.

A preferred embodiment of the present invention has the following formula:



Formula No. 1

wherein m and n are 1 to 5;

X designates a terminal carboxy acid, amide or alcohol group;

R^{249} is Trp, (L) or (D)Lys, (L) or (D) Tyr or (D)Phe;

R^{250} is Arg;

R^{251} is (L) or (D)Leu or Lys;

R^{252} is (L) or (D)Arg;

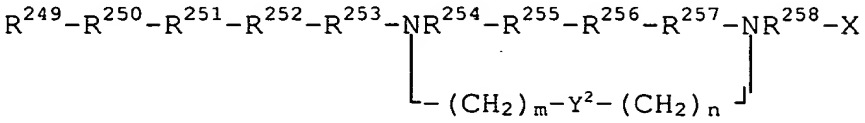
R^{253} is (D)- or (L)- Phe;

R^{254} is Ala;

R^{255} is (D)- or (L)- Leu or is Lys;

R^{256} is absent or is (L) or (D) Arg;
R^{257} is (L) or (D) Tyr;
R^{258} is Ala; and
Y^2 is amide, thioether, thioester or disulfide.

A more preferred embodiment of the present invention has the following formula:



Formula No. 1

wherein m and n are 1 to 5;
X designates a terminal carboxy acid, amide or alcohol group;
R^{249} is Trp, (D)Lys or (D)Phe;
R^{250} is Arg;
R^{251} is Lys or (D)Leu;
R^{252} is (D)Arg;
R^{253} is (D)- or (L)- Phe;
R^{254} is Ala;
R^{255} is (D)- or (L)- Leu;
R^{256} is absent or is Arg;
R^{257} is (D) Tyr;
R^{258} is Ala; and
Y^2 is amide, thioether, thioester or disulfide.

The currently most preferred backbone cyclized IL-6 antagonists of the invention are as follows:
Trp-Arg-Lys-(D)Arg-Phe-AlaC3-Leu-Arg-(D)Tyr-AlaN3-NH_2
designated herein as PTR-5045
(D)Lys-Arg-(D)Leu-(D)Arg-(D)Phe-AlaC3-(D)Leu-Arg-(D)Tyr-AlaN3-NH_2 designated herein as PTR-5041
(D)Phe-Arg-(D)Leu-(D)Arg-(D)Phe-AlaC3-Leu-(D)Tyr-AlaN3-NH_2
designated herein as PTR-5043

These peptide analogs were found to inhibit the cytotoxic effect of IL-6 in various *in-vitro* bioassays. PTR 5045 and PTR 5041 block IL-6 activity in F10.9 melanoma cells assay at concentration of 250 nM. These backbone cyclized peptide analogs inhibit the *in-vitro* binding of mutant IL-6-IL-6R chimera to gp130.
PTR 5045 was also found to be active *in-vivo* in prevention of body weight lose in acute phase response in mice(example 3), and to be metabolically functional bio-stable for 24 hours in renal homogenate.

The most striking advantages of these methods are:
1) Cyclization of the peptide sequence is achieved without compromising any of the side chains of the peptide thereby decreasing the chances of sacrificing functional groups essential for biological recognition and function.
2) Optimization of the peptide conformation is achieved by allowing permutation of the bridge length, direction, and bond type (e.g., amide, disulfide, thioether, thioester, etc.) and position of the bond in the ring.
3) When applied to cyclization of linear peptides of known activity, the bridge can be designed in such a way as to minimize interaction with the active region of the peptide and its cognate receptor. This decreases the chances of the cyclization arm interfering with recognition and function, and also creates a site suitable for attachment of tags such as radioactive tracers, cytotoxic drugs, light capturing substances, or any other desired label.

Preferably, the backbone cyclized peptide analogs of the present invention incorporates two such N^{α}-ω-functionalized amino acid derivatives which may be linked to one another to form N-backbone to N-backbone cyclic peptide analogs. Additional preferred analogs of the invention can be constructed with two or more cyclizations, including N-backbone to N-backbone, as well as backbone to side-chain or any other

peptide cyclization.

Backbone cyclized analogs of the present invention may be used as pharmaceutical compositions and in methods for the treatment of disorders including: cancers (including multiple myeloma/plasmacytoma), autoimmune diseases (including rheumatoid arthritis, multiple sclerosis, SLE and diabetes), infectious diseases (bacterial and viral infection, septic shock), inflammatory diseases (including pancreatitis), immune deficiency diseases (including AIDS), hematologic diseases (e.g., plasma cell dyscrasias, leukemia, lymphoma), allergic diseases, organ transplantation reactions, Castelman's disease, Lennart's T-cell lymphoma, Non-Hodgkin's lymphoma, Cardiac myxoma, mesangial proliferative glomerulonephritis, polyclonal B-cell activation conditions, abnormal acute phase protein production conditions.

The pharmaceutical compositions comprising pharmacologically active backbone cyclized IL-6 antagonist and a pharmaceutically acceptable carrier or diluent represent another embodiment of the invention, as do the methods for the treatment of a mammal in need thereof with a pharmaceutical composition comprising an effective amount of an IL-6 antagonist according to the invention. Methods of treatment using the compositions of the invention are useful for therapy of cancers (including multiple myeloma/plasmacytoma), autoimmune diseases (including rheumatoid arthritis, multiple sclerosis, SLE and diabetes), infectious diseases (bacterial and viral infection, septic shock), inflammatory diseases (including pancreatitis), immune deficiency diseases (including AIDS), hematologic diseases (e.g., plasma cell dyscrasias, leukemia, lymphoma), allergic diseases, organ transplantation reactions, Castelman's disease, Lennart's T-cell lymphoma, Non-Hodgkin's lymphoma, Cardiac myxoma, mesangial proliferative glomerulonephritis, polyclonal B-cell activation conditions, abnormal acute phase protein production conditions, using such compositions. The

pharmaceutical compositions according to the present invention advantageously comprise at least one backbone cyclized peptide analog which includes at least one D-isomer of amino acids in its sequence. These pharmaceutical compositions may be administered by any suitable route of administration, including topically or systemically. Preferred modes of administration include but are not limited to parenteral routes such as intravenous and intramuscular injections, as well as via nasal or oral ingestion.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing depicting known active IL-6 inhibitory and non-active peptides derived from the IL-6R.

Figure 2 describes the effect of backbone cyclic peptide analogs on B16.F10.9 melanoma cells growth.

Figure 3 describes in-vivo effects of IL-6 antagonists on IL-6 mediated acute-phase responses: a) IL-6 serum levels, b) fibrinogen plasma levels and c) changes in body weight, in normal and IL-6 knockout mice.

DETAILED DESCRIPTION OF THE INVENTION

Definitions:

The compounds herein described may have asymmetric centers. All chiral, diastereomeric, and racemic forms are included in the present invention. Many geometric isomers of olefins and the like can also be present in the compounds described herein, and all such stable isomers are contemplated in the present invention.

By "stable compound" or "stable structure" is meant herein a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and

formulation into an efficacious therapeutic agent.

As used herein and in the claims, "alkyl" or "alkylenyl" is intended to include both branched and straight-chain saturated aliphatic hydrocarbon groups having one to ten carbon atoms; "alkenyl" is intended to include hydrocarbon chains of either a straight or branched configuration having two to ten carbon atoms and one or more unsaturated carbon-carbon bonds which may occur in any stable point along the chain, such as ethenyl, propenyl, and the like; and "alkynyl" is intended to include hydrocarbon chains of either a straight or branched configuration having from two to ten carbon atoms and one or more triple carbon-carbon bonds which may occur in any stable point along the chain, such as ethynyl, propynyl, and the like.

As used herein and in the claims, "aryl" is intended to mean any stable 5- to 7-membered monocyclic or bicyclic or 7-to 14-membered bicyclic or tricyclic carbon ring, any of which may be saturated, partially unsaturated or aromatic, for example, phenyl, naphthyl, indanyl, or tetrahydronaphthyl tetralin, etc.

As used herein and in the claims, "alkyl halide" is intended to include both branched and straight-chain saturated aliphatic hydrocarbon groups having the one to ten carbon atoms, wherein 1 to 3 hydrogen atoms have been replaced by a halogen atom such as Cl, F, Br, and I.

As used herein and in the claims, the phrase "therapeutically effective amount" means that amount of novel backbone cyclic peptide analog or composition comprising same to administer to a host to achieve the desired results for the indications described herein, such as but not limited of inflammation, cancer, endocrine disorders and gastrointestinal disorders.

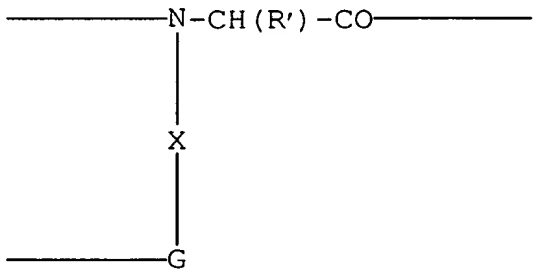
The term, "substituted" as used herein, means that any one or more hydrogen atoms on the designated atom is replaced with a selection from the indicated group, provided that the

designated atom's normal valency is not exceeded, and that the substitution results in a stable compound.

When any variable (for example R, X, Z, etc.) occurs more than one time in any constituent or in any Formula herein, its definition on each occurrence is independent of its definition at every other occurrence. Also, combinations of substituents and/or variables are permissible only if such combinations result in stable compounds.

As used herein "peptide" indicates a sequence of amino acids linked by peptide bonds. The IL-6 peptide antagonists of this invention comprise a sequence of amino acids of 4 to 24 amino acid residues, preferably 6 to 16 residues, each residue being characterized by having an amino and a carboxy terminus.

A "building unit" indicates an N^{α} derivatized α amino acid of the general Formula No. 2:



Formula No. 2

wherein X is a spacer group selected from the group consisting of alkylene, substituted alkylene, arylene, cycloalkylene and substituted cycloalkylene; R' is an amino acid side chain, optionally bound with a specific protecting group; and G is a functional group selected from the group consisting of amines, thiols, alcohols, carboxylic acids and esters, and alkyl halides; which is incorporated into the peptide sequence and subsequently selectively cyclized via the functional group G with one of the side chains of the amino acids in said peptide sequence or with another ω-functionalized amino acid

derivative.

The methodology for producing the building units is described in US Patent No. 5,883,293 and International Patent Applications WO 95/33765 and WO 98/04583, both of which are expressly incorporated herein by reference thereto for further details of this methodology. The building units are abbreviated by the three letter code of the corresponding modified amino acid followed by the type of reactive group (N for amine, C for carboxyl), and an indication of the number of spacing methylene groups. For example, Gly-C2 describes a modified Gly residue with a carboxyl reactive group and a two carbon methylene spacer, and Phe-N3 designates a modified phenylalanine group with an amino reactive group and a three carbon methylene spacer.

As used herein "backbone cyclic peptide" or "backbone cyclized peptide" denotes an analog of a linear peptide which contains at least one building unit that has been liked to form a bridge via the alpha nitrogen of the peptide backbone to another building unit, or to another amino acid in the sequence.

As used herein a "PTR" number denotes a reference number assigned to a backbone cyclic peptide analog that is synthesized, purified and fully characterized (e.g., by HPLC, MS, capillary electrophoresis, by amino acid analysis for peptide content and amino acid ratio determination).

As used herein and in the claims, in the formulae of the more preferred backbone cyclic peptide analogs, the superscript numbers following the amino acids refer to their position numbers in the native IL-6R.

Abbreviations:

Certain abbreviations are used herein to describe this invention and the manner of making and using it.

For instance, AcOH refers to acetic acid, Ada refers to adamantanacetyl, Adac refers to adamantanecarbonyl, Alloc refer

to allyloxycarbonyl, AIDS refers to acquired immune deficiency syndrome, Boc refers to the t-butyloxycarbonyl radical, BOP refers to benzotriazol-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate, BSA refers to bovine serum albumin, Cbz refers to the carbobenzyloxy radical, CNTF refers to ciliary neurotrophic factor, DCC refers to dicyclohexylcarbodiimide, DCM refers to Dichloromethane, Dde refers to 1-(4,4-dimethyl2,6-dioxocyclohex-1-ylidene-ethyl), DIEA refers to diisopropyl-ethyl amine, DMF refers to dimethyl formamide, DPPA refers to diphenylphosphoryl azide, Dtc refers to 5,5-dimethylthiazolidine-4-carboxylic acid, EDT refers to ethanedithiol, ESI-MS refers to electrospray ionization mass spectrometry, Fmoc refers to the fluorenylmethoxycarbonyl radical, HBTU refers to 1-hydroxybenztriazolyltetramethyl-uronium hexafluorophosphate, HF refers to hydrofluoric acid, HOBT refers to 1-hydroxybenzotriazole, HPLC refers to high pressure liquid chromatography, IL-1 refers to interleukin-1, IL-6 refers to interleukin-6, IL-6R refers to interleukin-6 receptor, IL-11 refers to interleukin-11, KS refers to Kaposi's sarcoma, LC-MS refers to liquid chromatography mass spectrometry, LIF refers to leukocyte inhibitory factor, LIF-R refers to leukocyte inhibitory factor receptor, LPS refers to lipopolysacaride, mAb refers to monoclonal antibody, MPS refers to multiple parallel synthesis, MS refers to mass spectrometry, NMM refers to N-methylmorpholine, NMP refers to 1-methyl-2-pyrolidonone, OSM refers to oncostatin M, PyBOP refers to Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate, PyBrOP refers to Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate, RA refers to Rheumatoid arthritis, RP refers to reverse phase, SLE refers to system lupus ethrythematosus, TBTU refers to 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, tBu refers to the tertiary butyl radical, TFA refers to trifluoroacetic acid, TIS refers to tri-isopropyl-silane.

The amino acids used in this invention are those which are available commercially or are available by routine synthetic methods. Certain residues may require special methods for incorporation into the peptide, and either sequential, divergent and convergent synthetic approaches to the peptide sequence are useful in this invention. Natural coded amino acids and their derivatives are represented by three-letter codes according to IUPAC conventions. When there is no indication, the L isomer was used. The D isomers are indicated by "D" before the residue abbreviation. List of Non-coded amino acids: Abu refers to 2-aminobutyric acid, Aib refers to 2-amino-isobutyric acid, β-Ala refers to β-Alanine,ChxGly refers to cyclohexyl Glycine, GABA refers to gama amino butyric acid, Hcys refer to homocystein, (p-Cl)Phe refers to para chloro Phenylalanine, (p-NH_2)Phe refers to para amino Phenylalanine, (p-F)Phe refers to para fluoro Phenylalanine, (p-NO_2)Phe refers to para nitro Phenylalanine, 1Nal refers to 1-naphthylalanine, 2Nal refers to 2-naphtylalanine, Nva refers to norvaline, Thi refers to thienylalanine.

As described earlier, IL-6 plays a pivotal role in mediating immune responses, acute-phase reactions and hematopoiesis. However, it has been shown that the loss of IL-6 regulation, or its overexpression, may be involved in a number of pathological conditions. Specifically, elevated IL-6 levels are detected in bacterial, parasite and viral infections, including HIV, as well as in chronic autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, psoriasis, and multiple sclerosis. In addition, IL-6 is implicated in the pathology of various neoplasms, such as multiple myeloma, leukemia, Kaposi's sarcoma, renal cell carcinoma and cardiac myxoma. In particular, IL-6 has been recognized as the major cytokine required for growth of multiple myeloma tumors and is also possibly involved in the n tumor-associated toxicity in multiple myeloma patients.

It is expected that peptides which inhibit IL-6 function will have broad therapeutic utility in many mammalian diseases. Therapeutic use of backbone cyclized IL-6 peptide antagonists of the present invention is expected to be beneficial in treating a variety of IL-6 associated diseases, many of which are currently treated with immunomodulators or immunosuppresants.

Such IL-6 associated diseases include:

a) Multiple myeloma/plasmacytoma.

b) Autoimmune diseased (including, but not limiting to, rheumatoid arthritis, multiple sclerosis, SLE and diabetes).

c) Infection diseases (bacterial and viral infection, septic shock).

d) Inflammatory diseases.

e) Immune deficiency diseases, including AIDS.

f) Hematologic diseases (e.g., plasma cell dyscrasias, leukemia, lymphoma).

g) Allergic diseases.

h) Organ transplantation reactions.

i) Castelman's disease.

j) Lennart's T-cell lymphoma

k) Non-Hodgkin's lymphoma.

l) Cardiac myxoma.

m) Mesangial proliferative glomerulonephritis.

n) Polyclonal B-cell activation conditions.

o) Abnormal acute phase protein production conditions.

By way of exemplification of the principles of the present invention, a search for inhibitory peptides was focused on the IL-6R/gp130 interface. This was followed by an investigation of the IL-6/IL-6R or the IL-6R/gp130 interfaces. According to one presently preferred embodiment the search comprises mainly rational design and massive screening using multiple parallel synthesis (MPS) approaches.

The advantages of backbone cyclic peptides over existing and previously suggested therapies of multiple myeloma, are as

follows:

The suggested IL-6 inhibitor is non-cytotoxic as compared with the currently utilized cytotoxic drugs. The effect of the IL-6 antagonist would be specific to multiple myeloma cells and a small subset of IL-6 dependent cells, where the other cytotoxic drugs are non-selective and kill all types of dividing cells in the body.

The suggested IL-6 antagonist is small and thus non-immunogenic by nature, as compared to the potentially immunogenic antibodies, antibody fragments and minibodies.

The suggested IL-6 antagonist could be modified to be orally bioavailable. It is unlikely that proteins (antibodies, fragments or minibodies) would be orally available.

Over 7400 individual backbone cyclized peptide analogs were synthesized in MPS format and tested by at least one type of bioassay for inhibition of IL-6 bioactivity.

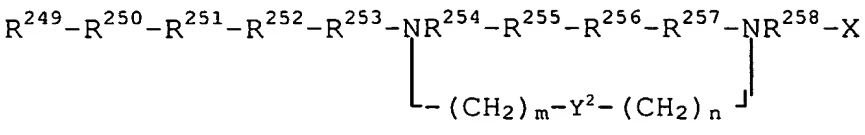
The best peptides achieved at this screening stage, demonstrated an estimated IC_{50} around 1 μM. This represent 10-100-times activity improvement of peptide analogs available at initiation of the research of the present invention (e.g. linear peptides derived from the IL-6R molecule).

About 30 peptide analogs (PTRs), chosen by activity from the MPS syntheses, were synthesized in large scale, purified and fully characterized. These PTRs were tested for IL-6 inhibitory activity in at least one in-vitro bioassay.

From the above described massive screening of backbone cyclized peptide analogs in MPS and PTR format three peptides were unexpectedly found as particularly active. These backbone cyclized peptide analogs mimic the IL-6R inhibitory domain of residues 249-258. But unlike the previously described peptides derived from this domain, these novel backbone cyclized peptide analogs possess unique features which make them more suitable for use in pharmaceutical compositions for treatment of pathological conditions associated with elevated levels of IL-6.

The preferred backbone cyclized IL-6 antagonists of the present invention are now described.

One embodiment has the following formula:



Formula No. 1

wherein m and n are 1 to 5;

X designates a terminal carboxy acid, amide or alcohol group;

R^{249} is Trp, (L) or (D)Lys, (L) or (D) Tyr or (D)Phe;

R^{250} is Arg;

R^{251} is (L) or (D)Leu or Lys;

R^{252} is (L) or (D)Arg;

R^{253} is (D)- or (L)- Phe;

R^{254} is Ala;

R^{255} is (D)- or (L)- Leu or is Lys;

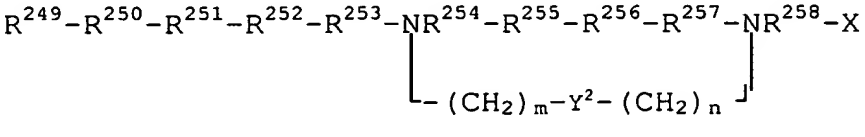
R^{256} is absent or is (L) or (D) Arg;

R^{257} is (L) or (D) Tyr;

R^{258} is Ala; and

Y^2 is amide, thioether, thioester or disulfide.

A currently more preferred embodiment has the following formula:



Formula No. 1

wherein m and n are 1 to 5;

X designates a terminal carboxy acid, amide or alcohol group;

R^{249} is Trp, (D)Lys or (D)Phe;

R^{250} is Arg;
R^{251} is Lys or (D)Leu;
R^{252} is (D)Arg;
R^{253} is (D)- or (L)- Phe;
R^{254} is Ala;
R^{255} is (D)- or (L)- Leu;
R^{256} is absent or is Arg;
R^{257} is (D) Tyr;
R^{258} is Ala; and
Y^2 is amide, thioether, thioester or disulfide.

A most preferred compound according to this embodiment is denoted PTR 5045 wherein the residues are as follows:

R^{249} is Trp;
R^{250} is Arg;
R^{251} is Lys;
R^{252} is (D)Arg;
R^{253} is Phe;
R^{254} is Ala;
R^{255} is Leu;
R^{256} is Arg;
R^{257} is (D)Tyr;
R^{258} is Ala; and
Y^2 is amide.

Another preferred compound according to this embodiment is denoted PTR 5041 wherein the residues are as follows:

R^{249} is (D)Lys;
R^{250} is Arg;
R^{251} is (D)Leu;
R^{252} is (D)Arg;
R^{253} is (D)Phe;
R^{254} is Ala;
R^{255} is Leu;
R^{256} is Arg;
R^{257} is (D) Tyr;
R^{258} is Ala; and

Y^2 is amide.

Another preferred compound according to this embodiment is denoted PTR 5043 wherein the residues are as follows:

R^{249} is (D)Phe;
R^{250} is Arg;
R^{251} is (D)Leu;
R^{252} is (D)Arg;
R^{253} is (D)Phe;
R^{254} is Ala;
R^{255} is Leu;
R^{256} is absent;
R^{257} is (D)Tyr;
R^{258} is Ala; and
Y^2 is amide.

The most preferred backbone cyclized IL-6 antagonists of the invention described in table 1:

Table 1: The most preferred analogs of the invention.

PTR 5045	Trp-Arg-Lys-(D)Arg-Phe-AlaC3-Leu-Arg-(D)Tyr-AlaN3-X
PTR 5041	(D)Lys-Arg-(D)Leu-(D)Arg-(D)Phe-AlaC3-(D)Leu-Arg-(D)Tyr-AlaN3-X
PTR 5043	(D)Phe-Arg-(D)Leu-(D)Arg-(D)Phe-AlaC3-Leu-(D)Tyr-AlaN3-X

where X is $-NH_2$ or -OH and the bridging group extends between the two building units.

A more preferred embodiment of the invention incorporates two N^{α}-ω-functionalized amino acid derivatives which may be linked to one another to form N-backbone to N-backbone cyclic peptide analogs.

Peptide analogs can be constructed with two or more cyclizations, including N-backbone to N-backbone, as well as backbone to side-chain or any other peptide cyclization.

The second cyclization, can be formed by incorporating at least

one additional building unit into a peptide sequence and linking it to another building unit, to the amino acid side chain or to any of the peptide terminals. In addition the second cyclization can be a side-chain to side-chain (including di-sulfide bond), or a side-chain to terminal cyclization.

It has now unexpectedly been found that the backbone cyclized IL-6 antagonists of the present invention which were identified by screening of individual backbone cyclized peptide analogs that were synthesized and assayed for inhibition of IL-6 activity, are 10-100 folds more active than the linear IL-6 inhibitory peptides previously described.

The backbone cyclic peptides of this invention are novel analogs which mimic the IL-6R inhibitory domain of residues 249-258. The amino acid sequence of the backbone cyclic analogs is based on what was identified as the most active inhibitory fragment of the IL-6R (Grube and Cochran, ibid).

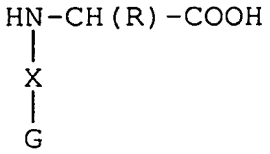
The present innovative backbone cyclic analogs preferably include 5 to 20 amino acids with special amino acid modifications. Specifically, at least one amino acid in each analog is a D-isomer of the amino acid.

The special feature of the novel backbone cyclic peptide analogs is their metabolic stability as tested *in vitro* against degradation of the most aggressive enzyme mixture in the body (e.g., renal homogenate). PTR 5045 is stable in these conditions for up to 24 hours. The previously described peptide analogs derived from the IL-6R, and fragments of the native protein, are significantly less stable metabolically.

Synthetic Approaches

According to the present invention peptide analogs are cyclized via bridging groups attached to the alpha nitrogens of amino acids that permit novel non-peptidic linkages. In general, the procedures utilized to construct such peptide analogs from

their building units rely on the known principles of peptide synthesis; most conveniently, the procedures can be performed according to the known principles of solid phase peptide synthesis. The innovation requires replacement of one or more of the amino acids in a peptide sequence by novel building units of the general Formula:



Formula No. 3

wherein R is the side chain of an amino acid, X is a spacer group and G is the functional end group by means of which cyclization will be effected. The side chain R is the side chain of any natural or synthetic amino acid that is selected to be incorporated into the peptide sequence of choice. X is a spacer group that is selected to provide a greater or lesser degree of flexibility in order to achieve the appropriate conformational constraints of the peptide analog. Such spacer groups include alkylene chains, substituted, branched and unsaturated alkylenes, arylenes, cycloalkylenes, and unsaturated and substituted cycloalkylenes. Furthermore, X and R can be combined to form a heterocyclic structure.

The terminal (ω) functional groups to be used for cyclization of the peptide analog include but are not limited to:

a. Amines, for reaction with electrophiles such as activated carboxyl groups, aldehydes and ketones (with or without subsequent reduction), and alkyl or substituted alkyl halides.

b. Alcohols, for reaction with electrophiles such as activated carboxyl groups.

c. Thiols, for the formation of disulfide bonds and reaction with electrophiles such as activated carboxyl groups, and alkyl or substituted alkyl halides.

d. 1,2 and 1,3 Diols, for the formation of acetals and ketals.

e. Alkynes or Substituted Alkynes, for reaction with nucleophiles such as amines, thiols or carbanions; free radicals; electrophiles such as aldehydes and ketones, and alkyl or substituted alkyl halides; or organometallic complexes.

f. Carboxylic Acids and Esters, for reaction with nucleophiles (with or without prior activation), such as amines, alcohols, and thiols.

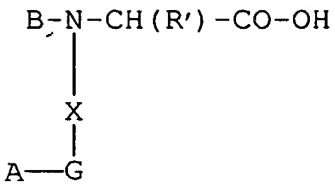
g. Alkyl or Substituted Alkyl Halides or Esters, for reaction with nucleophiles such as amines, alcohols, thiols, and carbanions (from active methylene groups such as acetoacetates or malonates); and formation of free radicals for subsequent reaction with alkenes or substituted alkenes, and alkynes or substituted alkynes.

h. Alkyl or Aryl Aldehydes and Ketones for reaction with nucleophiles such as amines (with or without subsequent reduction), carbanions (from active methylene groups such as acetoacetates or malonates), diols (for the formation of acetals and ketals).

i. Alkenes or Substituted Alkenes, for reaction with nucleophiles such as amines, thiols, carbanions, free radicals, or organometallic complexes.

j. Active Methylene Groups, such as malonate esters, acetoacetate esters, and others for reaction with electrophiles such as aldehydes and ketones, alkyl or substituted alkyl halides.

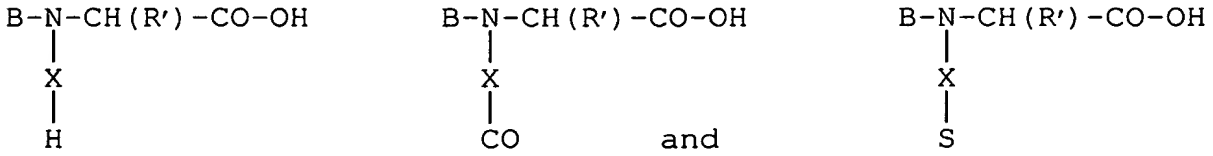
These backbone cyclized IL-6 peptide antagonists are prepared by incorporating at least one N^{α}-ω-functionalized derivative of an amino acids into a peptide sequence and subsequently selectively cyclizing the functional group with one of the side chains of the amino acids in the peptide sequence or with another ω-functionalized amino acid derivative. The N^{α}-ω-functionalized derivative of amino acids preferably have the following formula:



Formula No. 4

wherein X is a spacer group selected from the group consisting of alkylene, substituted alkylene, arylene, cycloalkylene and substituted cycloalkylene; R' is an amino acid side chain, optionally bound with a specific protecting group; B is a protecting group selected from the group consisting of alkyloxy, substituted alkyloxy, or aryl carbonyls; and G is a functional group selected from the group consisting of amines, thiols, alcohols, carboxylic acids and esters, aldehydes, alcohols and alkyl halides; and A is a specific protecting group of G.

Preferred building units are the co-functionalized amino acid derivatives wherein X is alkylene; G is a thiol group, an amine group or a carboxyl group; R' is phenyl, methyl or isobutyl; with the proviso that when G is an amine group, R' is other than H. Further preferred are ω-functionalized amino acid derivatives wherein R' is protected with a specific protecting group.
More preferred are ω-functionalized amino acid derivatives wherein G is an amino group, a carboxyl group, or a thiol group of the following formulae:



Formula No. 5 Formula No. 6 Formula No. 7

wherein X, R' and B are as defined above.

It will be appreciated that during synthesis of the peptide these reactive end groups, as well as any reactive side chains, must be protected by suitable protecting groups.

Suitable protecting groups for amines are alkyloxy, substituted alkyloxy, and aryloxy carbonyls including, but not limited to, tert butyloxycarbonyl (Boc), Fluorenylmethyloxycarbonyl (Fmoc), Allyloxycarbonyl (Alloc) and Benzyloxycarbonyl (Z).

Carboxylic end groups for cyclizations may be protected as their alkyl or substituted alkyl esters or thio esters or aryl or substituted aryl esters or thio esters. Examples include but are not limited to tertiary butyl ester, allyl ester, benzyl ester, 2-(trimethylsilyl)ethyl ester and 9-methyl fluorenyl.

Thiol groups for cyclizations may be protected as their alkyl or substituted alkyl thio ethers or disulfides or aryl or substituted aryl thio ethers or disulfides. Examples of such groups include but are not limited to tertiary butyl, trityl(triphenylmethyl), benzyl, 2-(trimethylsilyl)ethyl, pixyl(9-phenylxanthen-9-yl), acetamidomethyl, carboxymethyl, 2-thio-4-nitropyridyl.

It will further be appreciated by the artisan that the various reactive moieties will be protected by different protecting groups to allow their selective removal. Thus, a particular amino acid will be coupled to its neighbor in the peptide sequence when the N^{α} is protected by, for instance, protecting group A. If an amine is to be used as an end group for cyclization in the reaction scheme the N^{ω} will be protected by protecting group B, or an ε amino group of any lysine in the sequence will be protected by protecting group C, and so on. The coupling of the amino acids to one another is performed as a series of reactions as is known in the art of peptide synthesis. Novel building units of the invention, namely the N^{α}-ω functionalized amino acid derivatives are incorporated into the peptide sequence to replace one or more of the amino acids. If only one such N^{α}-ω functionalized amino acid derivative is selected, it will be cyclized to a side chain of another amino acid in the sequence or to either of the two

terminal amino acids of the peptide sequence. For instance: (a) an N^{α}-(ω-amino alkylene) amino acid can be linked to the carboxyl group of an aspartic or glutamic acid residue; (b) an N^{α} -(ω-carboxylic alkylene) amino acid can be linked to the ε-amino group of a lysine residue; (c) an N^{α}-(ω-thio alkylene) amino acid can be linked to the thiol group of a cysteine residue; and so on. A more preferred embodiment of the invention incorporates two such N^{α}-ω-functionalized amino acid derivatives which may be linked to one another to form N-backbone to N-backbone cyclic peptide analogs. Three or more such building units can be incorporated into a peptide sequence to create bicyclic peptide analogs as will be elaborated below. Thus, peptide analogs can be constructed with two or more cyclizations, including N-backbone to N-backbone, as well as backbone to side-chain or any other peptide cyclization.

As stated above, the procedures utilized to construct IL-6 antagonists of the present invention from novel building units generally rely on the known principles of peptide synthesis. However, it will be appreciated that accommodation of the procedures to the bulkier building units of the present invention may be required. Coupling of the amino acids in solid phase peptide chemistry can be achieved by means of a coupling agent such as but not limited to dicyclohexycarbodiimide (DCC), bis(2-oxo-3-oxazolidinyl) phosphinic chloride (BOP-Cl), benzotriazolyl-N-oxytrisdimethyl-aminophosphonium hexafluoro phosphate (BOP), 1-oxo-1-chlorophospholane (Cpt-Cl), hydroxybenzotriazole (HOBT), or mixtures thereof.

It has now been found that coupling of the subsequent amino acid to the bulky building units of the present invention may require the use of additional coupling reagents including, but not limited to: coupling reagents such as PyBOP (Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate), PyBrOP (Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate), HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3- tetramethyluronium

hexafluoro-phosphate), TBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate).

Novel coupling chemistries may be used, such as pre-formed urethane-protected N-carboxy anhydrides (UNCA'S), pre-formed acyl halides most preferably acyl chlorides.

Advantageously, it is also possible to use in situ generated amino acid chlorides.

Such coupling may take place at room temperature and also at elevated temperatures, in solvents such as toluene, DCM (dichloromethane), DMF (dimethylformamide), DMA (dimethylacetamide), NMP (N-methyl pyrrolidinone), dioxane, tetrahydrofuran, diglyme and 1,3 dichloropropane, or mixtures of the above.

General method for synthesis, purification and characterization of backbone cyclic peptides.

Synthesis:

Resin: 1 g Rink amide or Tenta-gel resin, with loading of 0.2-0.7 mmol/gr.

Fmoc-deprotection performed with 7 mL of 20% piperidine in NMP. Twice for 15 minutes following 5 washes with 10 ml NMP for 2 minutes with shaking.

Couplings:

1. Regular couplings (coupling to simple amino acids): with a solution containing 3 equivalents amino acid, 3 equivalents PyBroP and 6 equivalents of DIEA in 7 ml NMP. For 0.5-2 hours with shaking. Coupling is monitored by ninhydrine test and repeated until the ninhydrine solution become yellow.
2. Coupling of His and Asn with a solution containing 5 equivalents DIC and 5 equivalents HOBT in 10 ml DMF.
3. Coupling to Gly building units: with a solution containing 3 equivalents amino acid, 3 equivalents PyBroP and 6 equivalents DIEA in 7 ml NMP. Twice for 1-4 hours with shaking.
4. Coupling to building units which are not Gly: with a solution containing 5 equivalents amino acid, 1.5 equivalents triphosgene and 13 equivalents collidine in 15 ml dioxane or

THF. Twice for 0.5-2 hours at 50°C with shaking.
Removal of the Allyl and Alloc protecting groups of the building units performed with 1.5 equivalents per peptide of $Pd(PPh_3)_4$ in 30 ml DCM containing 5% acetic acid and 2.5% NMM. For 1-4 hours with shaking.
Cyclization performed with a solution containing 3 equivalents PyBOP and 6 equivalents DIEA in 7 ml NMP. For 0.5-2 hours with shaking. Cyclization is monitored by ninhydrine test and repeated if necessary.
Cleavage performed using 82%-95% TFA supplemented with scavengers: 1-15% H_2O, 1-5% TIS and 1-5% EDT.
Purification:
An individual purification method for each backbone cyclic peptide is developed on analytical HPLC to give the maximum isolation of the cyclic peptide from other crude components. The analytical method is usually performed using a C-18 Vydac column 250X4.6 mm as the stationary phase and water/ACN containing 0.1% TFA mixture gradient.
The preparative method is designed by implying the analytical separation method on the 2" C-18 Vydac preparative method. During the purification process, the peak containing the cyclic peptide is collected using a semi-automated fraction collector. The collected fractions are injected to the analytical HPLC for purity check. The pure fractions are combined and lyophilized.
Characterization:
The combined pure lyophilized material is analyzed for purity by HPLC, MS and capillary electrophoresis and by amino acid analysis for peptide content and amino acid ratio determination.

General method for synthesis, purification and characterization and screening of backbone cyclic peptides in MPS format.

The MPS procedure is used as the routine peptide development procedure. Individual peptides, or groups of a few peptides, are synthesized in 96-wells microtiter plates equipped with filters that allow passage of solvent but not of solid phase matrix. A simple and efficient valve apparatus that enable

simultaneous closing and opening of all the valves (produced by Millipore) is used. The system utilizes an approach in which each well is equipped with a solvent permeable membrane at the bottom that does not pass particles above a certain size. The process allow to place resin in the wells, perform reaction in solvent, and remove the solvent from all the wells simultaneously by applying vacuum. These special plates, which are available in the standard 96 well format allow the parallel synthesis of 96 peptides simultaneously. Parallel processing of 8 plates, performed manually, allows the synthesis of 768 individual peptides in a single experiment. The synthesis scale of the procedure is in the range of 1-5 μmole per well. Following purification by C18 reverse phase columns (SepPak purification), which is also carried in the standard 96 well format, the peptides are routinely dissolved in 1 ml of water to yield a theoretical crude concentration of 1-5 mM (depending of synthesis scale). Monitoring of chemical quality of the resulting peptides is performed by ESI-MS analysis. Analysis of several plates prepared on different occasions by different operators indicated a general success rate of about 80% as judged by the presence of the desired peptide mass in the crude preparation. Further analysis of a peptides from MPS is carried out by LC-MS. The analysis revealed crude peptide quality similar to crud preparations of peptides synthesized individually in large scale. Different steps or the complete process is now performed automatically using automatic peptide synthesizers. Peptides are tested for bioactivity at a dilution of 1:40 (theoretical crude concentration of 125 μM), or higher, apparently eliminated most all of the toxic effects and enabled routine biological testing of peptides. The actual concentration of the peptide in the preparation is not known therefore we assume a yield of about 10% of the theoretical value.

Detailed procedure for synthesis in MPS format:

For capacity of 5 μmole 10 mg resin with a substitution of 0.5

mmol/gr is used.

Fmoc deprotection: To each well 100 μl of 20% piperidine in NMP are added. The reaction shacked for 15 min. The NMP is removed by suction.

Washing after Fmoc deprotection: the resin is washed by placing 150 μl NMP into each well followed by evacuation of the solution by vacuum. This process is repeated 4 times.

Coupling using PyBroP:

Well capacity: 5 μmol

Amount of amino acid per coupling per well: 26 μmol

Amino acid in NMP concentration: 650 mM

Amino acid volume used: 40 μl

PyBroP amount: 26 μmol

PyBroP concentration: 403 mg/ml

PyBroP volume used: 30 μl

DIEA added: 10 μl

Total reaction volume: 80 μl

The amino acids are added to the pre-activation plate, then a fresh solution of PyBroP is distributed into this plate followed by addition of DIEA. The solution from this plate is transferred to the reaction plate and shacked for 1 hour. This coupling is repeated twice.

Coupling using Mukayama reagent:

Amino acid solution at 650 mM – 40 μl

Mukayama reagent at 111 mg/ml- 60 μl

Collidine added per well- 15 μl

The same procedure as for coupling with PyBroP. Reaction temperature 50°C, reaction time: first coupling 4h, second coupling 16h.

Allyl Alloc deprotection: this step is performed after completing the assembly, by addition of 180 μl solution of 1.5 g $Pd(PPh_3)_4$ in 20 ml CH_2Cl_2 containing 5% AcOH + 2.5% NMM.

Cyclization- this step is performed by addition of 100 μl solution of PyBoP in NMP + DIEA.

Cleavage of the peptide from the resin and SepPak purification: After final Fmoc deprotection the resin is transferred into a deep well microtiter plate, to each well 300 µl of TFA solution containing 2.5% TIS, 2.5% H_2O, 2.5% EDT are added. Removal of the TFA is performede by lyophilization. After cleavage the peptides are purified by SepPak.

Screening of IL-6 antagonists for biological activity.

In vitro bioassays

Screening for bioactivity of potential IL-6 inhibitory peptides was performed in vitro. Inhibition of IL-6 results in the death of IL-6 dependent cell lines such as the murine T1165 and B9, or the human TF1 and XG1. Alternatively, inhibition of IL-6 can be monitored by following the IL-6 induced differentiation of A375, B16.F10.9 and M1 cells which results in continued growth of the cells. Following IgG secretion by CESS cell line can be also used for monitoring IL-6 inhibitory activity.
Five types of in vitro bioassays for IL-6 inhibition using different murine and human cell lines were used in different stages of the search, for screening of inhibitory peptides.

Bioassay using B9 cells

B9 (murine myeloma) cells require IL-6 for growth. Inhibition of IL-6 results in cell death. The assay procedure was performed as described in Halimi et al. (ibid).

Bioassay using T1165 cells

T1165 (murine myeloma cells) require IL-6 for growth and die if IL-6 is omitted or inhibited. The validity of the assay was demonstrated by inhibition of IL-6 bioactivity using rabbit IL-6 antiserum.

Bioassay using B16.F10.9 melanoma cells

The F10.9 sub-line of B16 (murine melanoma) expresses gp130 but not IL-6R. Addition of human IL-6 and human IL-6R, or a chimera of IL-6/IL-6R results in differentiation which is associated

with growth arrest of the cells. Inhibition of IL-6 results in continued growth. Since the end point of this assay is cell growth rather than cell death, this assay system was used primarily in order to differentiate between suspected toxic effects observed on B9 or T1165 cells, and true inhibition of IL-6 activity.

The B16.F10.9 cell assay was routinely used as a confirmation assay for the T1165 or TF1 assay which were the primary screening assays (at different stages of the search).

Evaluation of the results in this assay is possible using two methods. One method uses vital dyes for monitoring cell growth. The second method comprises visual observation of the cell morphology and establishing a cut off point based on the following observations:

Cells which are not treated with IL-6 create a monolayer that covers almost all the surface area. Treatment with IL-6 causes growth arrest and a morphological change: the cells become very narrow and elongated as compared to the more spread out shape of the non-treated cells. Treatment of the cells with IL-6 and an inhibitory peptide apparently completely restores cell growth but does not completely restores the cell morphology. Treatment of cells with IL-6 and a non-inhibitory peptide results in cells that appear to be very similar to the IL-6 treated cells.

Based on such observations, the results of the assay are reported as the last concentration of the peptide that cause almost complete inhibition of the effect of IL-6.

Bioassay using A375 cells

The A375 are human melanoma cells. IL-6 induces differentiation of A375 cells which is associated with growth arrest similar to the phenomenon observed with the B16F10.9 murine melanoma cells. Thus, inhibition of IL-6 results in continued growth of the cells which is easily quantifiable. In such case toxicity of peptides would register as a false negative rather than false positive. This conditions is preferable during screening process. Second, since these are human cells, the molecules

involved in the bio-response, i.e. IL-6, IL-6R and gp130 are all of human origin thus ensuring authenticity of the tested peptide bioactivity. Another advantage of the A375 cells is their ability to be induced by other cytokines that share the gp130 signal transducer, i.e. leukemia inhibitory factor (LIF) and oncostatine M (OSM). As can be seen in table 2, LIF alone does not affect A375 cells, unless the respective receptor (LIF-R) is also added. Apparently the A375 line we use lacks LIF-R. OSM is highly effective alone. The availability of this assay system, allows us to test, the specificity of our IL-6 inhibitory peptides *vs.* other cytokines of the family, in an array of assays all using a single cell line. Assay performance is described in Savino et al. (ibid).

Table 2: Inhibition of A375 by cytokines other than IL-6

Cytokine	**Concentration (ng/ml)**	**% Inhibition (relative to IL-6)***
LIF	1	1
LIF	10	2
LIF + LIFR	10	45
OSM	1	94
OSM	10	141

* The maximal (plateau) inhibitory activity of IL-6 on A375 cells was taken as 100%

The assay was validated using rabbit IL-6 antiserum which was used to inhibit the bioactivity of IL-6. Testing of crude peptide preparations, as well as high concentrations of purified peptides resulted in marked toxicity to the cells. Further results indicated that the toxicity is dose dependent and decreases with increasing dilution of the crude peptide preparation, or at low concentration of the purified peptides. It is therefore anticipated that the assay can be used for screening of analogs with nM activity. It can be also used for specifically screening for demonstration of peptide selectivity in the context of gp130 related cytokines in order to

differentiate between peptides that inhibit IL-6 alone and peptides that inhibit IL-6 and additional, gp130 using cytokines.

Bioassay using TF1 cells

TF1 cells (human erythroleukemia) cells require IL-6 for growth and thus inhibition of IL-6 bioactivity results in cell death, similar to T1165 cells. The origin of all relevant molecules is human.

The problem associated with toxicity (false positive results), could be overcome in the TF1 based assay since these cells can be induced by additional cytokines which are unrelated to IL-6 (not using the gp130 signal transducer). It is possible to induce the cells to grow with IL-6 and with additional cytokine e.g. GM-CSF. Specific inhibition of IL-6 bioactivity is therefore expected to register only in cells induced by IL-6 and not with GM-CSF. Validity of the use of TF1 cells and of the differential inhibition concept was demonstrated by the use of rabbit IL-6 antiserum. Only the IL-6 induced growth was inhibited by the antiserum. Assay performance is described in Fourcin et al. (ibid).

Inhibition of the IL-6 using peptides derived from the IL-6R or from gp130, but not from the IL-6 itself could result in inhibition of other cytokines that also utilize the gp130 signal transduction system. In order to test the specificity of the peptide analogs in that respect, bioassays for the bioactivity of some of the other cytokines in the groups (i.e. IL-11, CNTF, OSM), are performed.

In vitro binding assays

Binding assays are intended to measure the direct effect of the peptide on formation of the IL-6 active hexamer. Unlike the bioassays, the use of this assay could clearly demonstrate the mode of activity of the tested peptides. A simple format for such assay would be as follows: IL-6, IL-6R and soluble gp130 would be mixed in solution together with the test peptide.

Capture of the putative hexamer would be achieved by an anti-gp130 antibody and detection of the bound complex would be achieved by antibody to either IL-6 or IL-6R. A separate assay performed in order to test the interference of the peptide in the IL-6/IL-6R interaction.

Assays of similar format are used for testing the inhibition specificity of the peptide to the IL-6 bioactive complex by replacing the IL-6-IL-6R complex with commercially available cytokines and receptors of the other cytokines known to utilize the gp130 signal transducer.

In-vivo Assays

The prime clinical target for the IL-6 antagonist is multiple myeloma. *In-vivo* model systems are performed in mice inoculated with murine IL-6 dependent myeloma cell lines. Nude mice grafted with human multiple myeloma cells are also used. Other *in-vivo* assays which are used for testing the inhibitory effects of the backbone cyclized IL-6 antagonists are: IL-6 mediated acute-phase response, IL-6 mediated adjuvant arthritis, and pancreatitis induced by taurocholic acid as described in the following examples.

The skilled artisan will appreciate that the following examples are merely illustrative and serve as non limitative exemplification of the principles of the present invention and that many variations and modifications are possible within the scope of the currently claimed invention as defined by the claims which follow.

EXAMPLES

Example 1. **Detailed synthesis of PTR 5045 (Trp-Arg-Lys-(D)Arg-Phe-AlaC3-Leu-Arg-(D)Tyr-AlaN3-NH_2).**

Two grams of Tenta-Gel resin (0.22 mmol/g), were swelled in NMP in a reaction vessel equipped with a sintered glass bottom and

placed on a shaker. All the Fmoc protecting groups were removed by reaction with 20% piperidine in NMP (2 times 10 minutes, 10 ml each) followed by NMP wash (5 times two minutes, 15 ml each). Fmoc removal was monitored by ultraviolet absorption measurement at 290 nm. The couplings of the amino acids: Fmoc-Arg(pmc)-OH, Fmoc-Leu-OH, Fmoc-(D)Arg(pmc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(pmc)-OH, Fmoc-Trp(Boc)-OH were carried out with 4 eq (1.76 mmol) of the amino acid +PyBrop (4 equivalents, 1.76 mmol)+ DIEA (8 equivalents, 3.52 mmol) in NMP (10 ml) for 1.5 hour at room temperature. Reaction completion was monitored by the qualitative ninhydrin test (Kaiser test). After each coupling, the peptide-resin was washed with NMP (5 times with 15 ml NMP, 2 minutes each). The coupling of Fmoc-(D)Tyr(t-Bu)-OH to AlaN3 building unit was carried out by use of 4eq of amino acid + a mixture of TPTU and ToPPyU(4 eq, 1.76 mmol) in 10 ml NMP + 8 eq DIEA, double coupling: first coupling 2h, second coupling overnight. The coupling of Fmoc-Phe-OH to AlaC3 building unit was carried out by the same manner. Coupling completion was monitored by HPLC . The Allyl/Aloc protecting groups were removed by reaction with $Pd(PPh_3)_4$ and acetic acid 5%, morpholine 2.5% in CH_2Cl_2, under argon, for 2 hours at room temperature. The peptide resin was washed with $CHCl_3$(3 times, 5 min 30 ML each) followed by NMP. Cyclization was carried out with PyBOP 3 equivalents, DIEA 6 equivalents, in NMP, at room temperature for 2h. Final Fmoc deprotection was carried out with 20% piperidine in NMP as above. The peptide resin was washed with CH_2Cl_2 and dried under reduced pressure. The peptide was cleaved from the resin by reaction with TFA 94%, water 2.5%, EDT 2.5%, TIS 1%, at 0°C for 15 minutes and 2 hours at room temperature under argon. The mixture was filtered and the resin was washed with a small volume of TFA. The filtrate was placed in a rotary evaporator and all the volatile components were removed. An oily product was obtained. It was triturated with ether and the ether decanted, three times. A white powder was obtained. This crude product was dried. The weight of the crude product was 400 mg. After purification by HPLC a single peak was obtained with 100%

purity as detected by analytical HPLC and capillary electrophoresis. The expected mass of 1489.7 daltons was detected by mass spectroscopy.

Example 2: **the effect of backbone cyclic peptide analogs on B16.F10.9 melanoma cells growth.**

Peptides were added to B16.F10.9 melanoma cells in the presence of 200 ng/ml IL-6 and 125 ng/ml sIL-6R. Incubation for three days. (Peptide concentration was calculated for average molecular weight of 1500 Da. Sequence of control peptides: PTR 5049: Trp-Arg-Lys-(D)Arg-Phe-AlaC3-Leu-Arg-Tyr-AlaN3-NH_2
PTR 4041: Lys-GlyC2-Leu-Ile-Gln-Leu-Phe-GlyN3-Lys-Lys-NH_2
The results described in figure 2 show that PTR 5045 and PTR 5041 fully block IL-6 activity at concentration of about 250 nM while PTR 5049 and PTR 4041 are not active.

Example 3: **The in-vivo effect of IL-6 antagonists on IL-6 mediated acute-phase response.**

The objective is to establish a simple first-line *in-vivo* model, for testing the activity of IL-6 inhibitors. The turpentine model was chosen based on the principal role that IL-6 plays in this inflammatory response.

Systemic and localized inflammation elicit a general reaction in the organism, known as the acute phase response, which includes fever, loss of body weight, hypoglycemia, and changes in the serum levels of several plasma proteins produced by the liver. IL-6 is an important mediator of the acute-phase reaction (see Heinrich et.al., Biochemistry J. l 265:621, 1990 for a comprehensive review), together with IL-1 and TNF-α. It has been shown previously that sterile tissue damage caused by injection of turpentine induces an acute-phase reaction, and that IL-6 is an essential mediator of this response (Rokita et.al., Cytokine 5:454, 1993).

To confirm the role of IL-6 in this phenomenon we used

IL-6-deficient mice generated by gene targeting (knock out mice).

Method and Results

IL-6 knock-out (-/-) and matched wild-type (C57/Black; CB) mice were used. Sterile tissue damage was induced by subcutaneous injection of turpentine, 0.1 ml, into both hind limbs. IL-6 was determined in the serum by ELISA (QuantikineM, R&D). For liver acute-phase proteins, Fibrinogen was determined in citrated plasma, by the calcium method.

IL-6 levels rose 7-fold following turpentine injection in normal, but not in IL-6 deficient mice (Figure 3a).

Fibrinogen levels rose 3- to 4-fold following turpentine injection in normal, but not in IL-6 deficient mice (Figure 3b).

Loss of body weight occurred following turpentine injection in normal, but not in IL-6 deficient mice (Figure 3c).

No changes in fibrinogen levels were detected 6 hours after turpentine injection. Elevated fibrinogen levels were observed at 12 hours post injection, and reach a maximal level by 24 hours post injection.

Conclusions

Our results confirm the previously published findings (Kozak et al., American J. Physiology 272:2 R621, 1997; Kopf et.al., Nature 368:339-342, 1994), which suggest that IL-6 is an essential mediator of the acute-phase response to turpentine. The turpentine model can serve as a first-line *in-vivo* assay to determine the pharmacological efficacy of anti-IL-6 treatments.

PTR 5045 was tested in this model in compare to the non-relevant control peptide PTR 4041 (Lys-GlyC2-Leu-Ile-Gln-Leu-Phe-GlyN3-Lys-Lys-NH_2). The results are summarized in the following table 3:

Table 3: The in-vivo effect of PTR 5045 on IL-6 mediated acute-phase response.

	Mean weight loss	
	%	grams
PTR 4041 (control)	-7.3	-2.51
PTR 5045	-3.93	-1.35
Student t-test	P=0.035	P=0.047
Mann-Whitney test	P=0.029	P=0.023

10 mice received 1 or 10 mg/kg of each peptide analog.

PTR 5045 reduces by about half the effect mediated by IL-6 on body weight during acute inflammation. The difference between PTR 5045 and the control peptide is significant also if taking only the 1 mg/kg dose.

Example 4: **IL-6 mediated adjuvant arthritis.**

The objective is to establish an *in-vivo* model of rheumatoid arthritis (RA), for testing the activity of Interleukin-6 (IL-6) inhibitors. The adjuvant arthritis model was chosen because it has clinical and pathological similarities to RA in humans, and based on the putative role that IL-6 plays in this inflammatory condition.

Rheumatoid arthritis is a chronic, multi-system autoimmune disease, mainly characterized by a persistent inflammatory synovitis, usually involving peripheral joints in a symmetric distribution. IL-6 has been implicated in RA, since increased levels were found in serum and synovial fluid of RA patients, and these levels were correlated with clinical parameters of inflammation (Miltenburg et al., British J. Rheumatology 30:186, 1991). Adjuvant induced arthritis in rats is used as a model for human RA because adjuvant arthritic animals develop the inflammatory and immunologic features which are observed in RA patients. Furthermore, IL-6 levels are elevated in the serum of arthritic rats, and the levels correlate with inflammation, and with the progress of the syndrome.

Method and Results

Genetically susceptible Lewis rats were used. The arthritis is induced by a single injection of complete Freund's adjuvant (CFA), containing heat killed Mycobacterium Tuberculosis in oil (10 mg/ml). The animals were injected intradermally at the base of the tail. The arthritis developed two weeks after immunization, and involved the small joints of the extremities. The rats were then subjected to a clinical scoring of the inflamed joints, and to a histo-pathological evaluation. The rate of success in inducing arthritis is greater than 85%. Female rats are more susceptible than males. Inflammation of the synovium, formation of pannus, erosion of cartilage and bone were all observed histopathologically, confirming the clinical severity.

Dexamethasone treatment completely abolished the clinical signs of arthritis, and can therefore be used as a positive control in this model. Delivery of the dexamethason treatment was successfully achieved by employing osmotic mini-pumps, which could also be used for the continuous administration of peptides.

Conclusions

Our results confirm the previously published descriptions of this RA model (Stanescu et al., Arthritis and Rheumatism 30:779, 1987).

The adjuvant arthritis model can serve as a disease model to determine the pharmacological activity of anti-IL-6 treatments. The testing is based on scoring of clinical parameters at the onset of the disease (day 12).

Example 5: **Pancreatitis Induced by Taurocholic Acid.**

The objective is to establish an *in-vivo* model of acute pancreatitis for testing the activity of IL-6 inhibitors. The taurocholic acid induced pancreatitis model has clinical and pathological similarities to severe necrotizing acute pancreatitis.

In its severe form acute pancreatitis has clear systemic manifestations such as: circulatory failure, metabolic acidosis, ascites, hyperglycemia, hyperlipidemia, and ultimately a multisystem organ failure. IL-6 has been implicated in acute pancreatitis, since elevated serum levels were more predictive of disease severity or lethality as compared with C-reactive protein in patients with acute pancreatitis (Leser et al., Gastroenterology 101:782:5, 1991). Taurocholic acid induced pancreatitis in rats is used as a model for human pancreatitis because pancreatitis animals develop the biochemical and pathological features which are observed in pancreatitis patients. Furthermore, IL-6 levels were elevated in the serum of pancreatitis rats.

Method and Results

Male Wistar rats were used. The pancreatitis was induced by the infiltration of 0.5 ml of 10% sodium taurocholate, into several sites of the pancreatic parenchyma with a 30G needle. A progressive detergent effects took place, which resulted in a diffuse pancreatic necrosis, and a high mortality rate. The measured parameters were serum levels of the pancreatic enzymes amylase and lipase, serum IL-6, and mortality. In addition, a histopathological evaluation of the pancreas was performed.
A mortality rate of 60-80% was found in the pancreatitis animals, with no mortality in the sham control group.
Severe hemorrhages and necrosis were evident in the pancreas, after taurocholate induction.
Fat necrosis throughout the peritoneal cavity, and marked intestinal dilation were found at 24 hours.
A significant elevation in serum IL-6 was observed at three hours, and reached its peak within 6 hours from the taurocholate injection.
A significant elevation of serum amylase was observed at two hours after pancreatitis induction.

Conclusions

Our results confirm the previously published findings, that

IL-6 is elevated in animal models of pancreatitis. The taurocholate induced pancreatitis model can serve as a disease model to determine the pharmacological activity of anti-IL-6 treatments. The testing is be based on measuring levels of pancreatic enzymes in the serum, mortality, and histopathological scoring.

Example 6: **Synthesis and in vitro activity of further preferred backbone cyclized IL-6 antagonist PTRs.**

Additional PTR analogs that were synthesized are listed in table 4, together with their respective chemical and biological data. Most of the peptides were synthesized during the initial phase of the research as part of the effort to discovery active peptides. The best IC_{50} were observed using the B16F10.9 cell assay are around 10 μ/ml for PTR-5005 and around 20 μg/ml for PTR-5037. Of these two peptides, only PTR-5005 appears to be active on TF1 cells.

Table 4: Summary of synthesis and bioactivity of further preferred PTRs.

PTR	TF1 inhibition (100 ug/ml)	T1165 inhibition (100 ug/ml)	B16F10.9 inhibition (33 ug/ml)	Sequence	Synt. scale (umole)	MW	Pure (mg)	% Purity (HPLC)
5001	3	16	0	Thr-GlyC3-Gln-Gly-Ala-Ala-Ile-Ile-GlyN3-Gln-Pro	460	1022	56.7	100
5003	20	32	36	Tyr-Arg-Leu-Arg-Phe-GlyC3-Leu-Arg-Tyr-GlyN2	460	1410	43.4	100
5005	98	132	37	Tyr-Arg-Leu-Arg-Phe-GlyC3-Leu-Arg-Tyr-GlyN2		1254	19.6	100
5007	6	13	0	Tyr-Arg-Leu-Arg-Phe-GlyN2-Leu-Arg-GlyC3-Arg	460	1403	39.4	100
5009	8	24	NT	GlyC3-Glu-Ser-Gln-Lys-GlyN3-Ala-Ala-Gln-Leu	460	1112	23.9	
5011	11	23	NT	Tyr-Arg-Leu-Ile-Phe-Glu-GlyN2-Arg-Tyr-GlyC2	230	1369	18.4	
5013	28	22	NT	Tyr-Arg-Leu-Arg-Phe-Glu-GlyN2-Arg-Tyr-GlyC2	230	1412	24.6	100
5015	21	19	NT	Tyr-Arg-Leu-Arg-Phe-Glu-GlyN2-Arg-Tyr-GlyC2		1086		
5017	30	NT	NT	Arg-Leu-Arg-Ala-Glu-GlyC2-Ser-Lys-GlyN3-Phe	460	1280	277.9	90
5019	14	NT	NT	Asp-Leu-Gln-GlyN3-Ser-Leu-Arg-Ala-Leu-Arg-Gln	460	1294	21.9	99
5021	20	NT	NT	Asp-Leu-Gln-GlyN2-Ser-Leu-Arg-Ala-Leu-Arg-Gln	460	1280	3.3	87
5023	11	NT	NT	Asp-Leu-Gln-GlyN2-Ser-Leu-Arg-Ala-Leu-Arg-Gln	460	1280	10.5	99
5025	28	NT	NT	Asp-Leu-Gln-GlyN2-Ser-Leu-Arg-Ala-Leu-Arg-Gln	460	1280	4	100
5027	56	NT	NT	Tyr-Arg-Leu-Phe-Arg-GlyC3-Leu-Arg-Tyr-GlyN2	460	1410	1.5	100
5029	36	NT	NT	Tyr-Arg-Leu-Arg-Phe-Glu-GlyN2-Arg-Tyr-GlyC2	460	1412	3.5	100
5031	20	NT	NT	Tyr-Arg-Lys-Arg-Phe-GlyN2-Leu-Arg-GlyC3-Arg	460	1419		98
5033	20	NT	NT	Tyr-Arg-Lys-Arg-Phe-GlyN2-Leu-Arg-GlyC3-Arg	460	1264	46.3	100
5035	2	NT	NT	Tyr-Arg-GlyN2-Arg-Phe-Glu-Leu-Arg-GlyC3-Arg	460	1419	60.2	100
5037	1	NT	NT	Tyr-Arg-Leu-Arg-Phe-AlaC3-Leu-Arg-Tyr-AlaN3-Ala-Glu	460	1636	86.7	99
5039	-14	NT	NT	Phe-Arg-Leu-Arg-Phe-AlaC3-Leu-Arg-Tyr-AlaN3	460	1436	40.6	95

NT - Not Tested

Lys	Arg	Lys	(D)Arg	Phe	AlaC3	Leu	Arg	BTyr	AlaN3	85	active at <12 uM
Trp	Arg	Lys	(D)Arg	Phe	AlaC3	Leu	Arg	BTyr	AlaN3	32	active at <12 uM
Trp	Arg	Lys	(D)Arg	(D)Phe	AlaC3	Leu	Arg	BTyr	AlaN3	41	active at <12 uM
(D)Lys	Arg	(D)Leu	(D)Arg	(D)Phe	AlaC3	(D)Leu	Arg	(D)Tyr	AlaN3	92	active at 47 uM
(D)Lys	Arg	Lys	Arg	(D)Phe	AlaC3	Lys	Arg	Tyr	AlaN3	55	active at 62 uM
(D)Lys	Arg	Lys	(D)Arg	(D)Phe	AlaC3	Leu	Arg	Tyr	AlaN3	13	active at 62 uM
Tyr	Arg	Lys	(D)Arg	(D)Phe	AlaC3	Leu	(D)Arg	BTyr	AlaN3	19	active at 47 uM
Lys	Arg	Lys	Arg	Phe	AlaC3	Lys	(D)Arg	BTyr	AlaN3	21	active at 62 uM
(D)Lys	Arg	Lys	(D)Arg	Phe	AlaC3	(D)Leu	Arg	Tyr	AlaN3	3	active at 62 uM
Lys	Arg	Leu	(D)Arg	(D)Phe	AlaC3	Lys	Arg	BTyr	AlaN3	22	not active
(D)Lys	Arg	Lys	Arg	Phe	AlaC3	Leu	Arg	(D)Tyr	AlaN3	25	not active
(D)Lys	Arg	Lys	Arg	(D)Phe	AlaC3	Leu	Arg	(D)Tyr	AlaN3	23	low activity at 62 uM
(D)Lys	Arg	Lys	Arg	(D)Phe	AlaC3	Lys	(D)Arg	(D)Tyr	AlaN3	17	low activity at 62 uM
(D)Lys	Arg	Lys	(D)Arg	(D)Phe	AlaC3	Lys	Arg	Tyr	AlaN3	17	not active at 62 uM
(D)Lys	Arg	Lys	Arg	Phe	AlaC3	Lys	Arg	(D)Tyr	AlaN3	21	not active
(D)Lys	Arg	Lys	Arg	Phe	AlaC3	Leu	(D)Arg	(D)Tyr	AlaN3	19	not active
(D)Lys	Arg	Lys	Arg	Phe	AlaC3	(D)Leu	(D)Arg	Tyr	AlaN3	27	not active
(D)Tyr	Arg	Lys	Arg	Phe	AlaC3	Lys	(D)Arg	(D)Tyr	AlaN3	22	not active

BTyr is a mixture of DTyr and LTyr, and thus these wells contain 2 peptides each.

The results in table 6 demonstrate a general agreement between the results of both types of bioassays. Most of the peptides tested which were positive on TF1 cells, were also positive on B16F10.9 cells.

The best peptide analogs obtained appear to inhibit the effect of IL-6 on B16F10.9 cells at theoretical crude concentrations below 12 uM, suggested IC_{50} values close to 1 uM. Somewhat higher IC_{50} values are observed in the TF1 assay.

THE CLAIMS

What is claimed is:

1. A backbone cyclized peptide analog having IL-6 antagonist activity, comprising a peptide sequence of five to twenty amino acids that incorporates at least one building unit, said building unit containing one nitrogen atom of the peptide backbone connected to a bridging group comprising an amide, thioether, thioester or disulfide, wherein the at least one building unit is connected via the bridging group to form a cyclic structure.

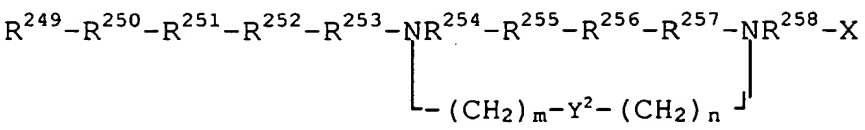
2. The backbone cyclized analog of claim 1 wherein the peptide sequence comprises six to twelve amino acids.

3. The backbone cyclized analog of claim 1 wherein the peptide sequence incorporates at least one D-isomer of an amino acid.

4. The backbone cyclized analog of claim 1 wherein the peptide sequence incorporates at least two D-isomers of an amino acid.

5. The backbone cyclized analog of claim 1 wherein the linear peptide sequence is derived from the IL-6 receptor.

6. The backbone cyclized analog of claim 1 having the general formula 1:



Formula No. 1

wherein m and n are 1 to 5;

X designates a terminal carboxy acid, amide or alcohol

group;

R^{249} is Trp, (L) or (D)Lys, (L) or (D) Tyr or (D)Phe;

R^{250} is Arg;

R^{251} is (L) or (D)Leu or Lys;

R^{252} is (L) or (D)Arg;

R^{253} is (D)- or (L)- Phe;

R^{254} is Ala;

R^{255} is (D)- or (L)- Leu or is Lys;

R^{256} is absent or is (L) or (D) Arg;

R^{257} is (L) or (D) Tyr;

R^{258} is Ala; and

Y^2 is amide, thioether, thioester or disulfide.

7. The backbone cyclized analog of claim 6 wherein

R^{249} is Trp, (L) or (D)Lys or (D)Phe;

R^{250} is Arg;

R^{251} is Lys or (D)Leu;

R^{252} is (D)Arg;

R^{253} is (D)- or (L)- Phe;

R^{254} is Ala;

R^{255} is (D)- or (L)- Leu;

R^{256} is absent or is Arg;

R^{257} is (D)Tyr;

R^{258} is Ala; and

Y^2 is amide, thioether, thioester or disulfide.

8. A backbone cyclized IL-6 antagonist of claim 6 having the formula:

Trp-Arg-Lys-(D)Arg-Phe-AlaC3-Leu-Arg-(D)Tyr-AlaN3-NH_2

9. A backbone cyclized IL-6 antagonist of claim 6 having the formula:

(D)Lys-Arg-(D)Leu-(D)Arg-(D)Phe-AlaC3-(D)Leu-Arg-(D)Tyr-AlaN3-NH_2

10. A backbone cyclized IL-6 antagonist of claim 6 having the formula:

(D)Phe-Arg-(D)Leu-(D)Arg-(D)Phe-AlaC3-Leu-(D)Tyr-AlaN3- NH_2

Further preferred analogs are described in table 5 and table 6.

Table 5: Further preferred backbone cyclic peptide analogs capable of inhibiting IL-6 derived from either IL-6, IL-6R or gp130.

No.	Origin	Sequence									
01	IL-6R	Tyr	Arg	Leu	Arg	Phe	GlyN2	Leu	Arg	Tyr	GlyC3
02	IL-6R	Tyr	Arg	Leu	Arg	Phe	GlyN2	Leu	Arg	GlyC3	Arg
03	IL-6R	Arg	Tyr	Arg	Ala	Glu	GlyC2	Ser	Lys	GlyN3	Phe
04	IL-6R	Ala	Glu	Arg	Ser	Lys	Thr	Phe	Thr	Thr	Trp
05	IL-6R	GlyN3	Arg	GlyC3	Lys	Thr	Phe	Thr	Thr	Trp	Met
06	IL-6R	Ala	GlyC2	D/LArg	Phe	Lys	DThr	Phe	D/LThr	GlyN3	D/LTrp
07	IL-6R	Ala	Val	Ala	Arg	GlyC2	Pro	Arg	Trp	Leu	GlyN2
08	IL-6R	Ala	Val	Pro	Glu	GlyC2	Asp	Ser	GlyN2	Phe	Ile
08	IL-6R	Ala	Val	Pro	Glu	GlyC2	Asp	Ser	GlyN2	Tyr	Ile
09	IL-6R	Glu	Gly	GlyC2	Ser	Ser	Phe	Tyr	GlyN2	Val	Ser
10	IL-6R	Tyr	Ile	Val	Ser	GlyC2	Ala	Val	Ala	Ser	GlyN2
11	IL-6R	GlyC2	Met	Ala	Val	Ala	Ser	Ser	Val	Gly	GlyN2
12	IL-6R	Ala	Val	GlyC2	Ser	Ser	Val	Gly	GlyN2	Lys	Phe
13	IL-6R	Gly	Ala	GlyC2	Ile	Leu	Gln	Pro	GlyN2	Pro	Pro
14	IL-6R	GlyC2	Gln	Pro	Asp	Pro	Pro	Ala	GlyN2	Ile	Thr
15	IL-6R	Ser	Gln	GlyC2	Ser	Gln	Lys	Phe	Ser	Ala	GlyN2
16	IL-6	GlyC2	Asn	Leu	Pro	Lys	Met	Ala	Glu	Lys	GlyN2
17	IL-6	GlyC2	Lys	Val	Leu	Ile	Gln	Phe	GlyN2	Gln	Lys
18	gp130	GlyC2	Asn	Phe	Thr	Leu	Lys	Ser	GlyN2	Trp	Ala
19	gp130	Phe	Ala	Asp	Ala	GlyC2	Ala	Lys	Arg	Asp	GlyN2
20	gp130	GlyC2	Thr	Pro	Thr	Ser	Ala	Thr	GlyN2	Asp	Tyr
21	gp130	Asn	Phe	Asp	Pro	GlyC2	Tyr	Lys	Val	Lys	GlyN2
22	gp130	Asn	Pro	GlyC2	His	Asn	Leu	Ser	Val	Ile	GlyN2
23	gp130	Ser	Ile	GlyC2	Lys	Leu	Thr	Trp	Thr	Asn	GlyN2
24	gp130	Tyr	Arg	GlyC2	Lys	Asp	Ala	Ser	GlyN2	Trp	Ser
25	gp130	Arg	Thr	Lys	Asp	Ala	Ser	GlyC2	Trp	Ser	GlyN2
26	gp130	GlyC2	Ile	Pro	Pro	Glu	Asp	Thr	Ala	Ser	GlyN2
27	gp130	GlyC2	Asp	Thr	Ala	Ser	Thr	Arg	Ser	Ser	GlyN2
28	gp130	GlyC2	Ala	Ser	GlyN2	Arg	Ser	Ser	Phe	Thr	Val
29	gp130	Ser	Phe	GlyC2	Val	Gln	Asp	Leu	Lys	Pro	GlyN2
30	gp130	Tyr	Val	Phe	Arg	Ile	Arg	GlyC2	Met	Lys	GlyN2

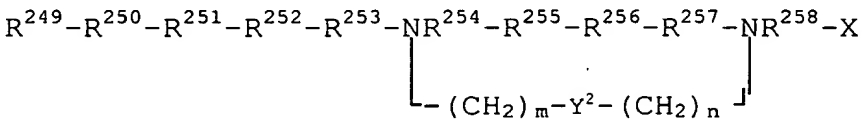
The average activity of the peptides found following the above procedure is estimated to be over 100 μM as estimated from the results of the MPS experiments.

Table 6: Summary of activity of further preferred analogs derived from the IL-6R.

Sequence	TF1	B16F10.9

11. A pharmaceutical composition comprising a backbone cyclized IL-6 antagonist comprising a peptide sequence of five to twenty amino acids that incorporates at least one building unit, said building unit containing one nitrogen atom of the peptide backbone connected to a bridging group comprising an amide, thioether, thioester or disulfide, wherein the at least one building unit is connected via the bridging group to form a cyclic structure, together with a pharmaceutically acceptable carrier or diluent.

12. The pharmaceutical composition of claim 11 wherein the IL-6 antagonist is a backbone cyclized peptide analog having the general formula 1:



Formula No. 1

wherein m and n are 1 to 5;

X designates a terminal carboxy acid, amide or alcohol group;

R^{249} is Trp, (L) or (D)Lys, (L) or (D) Tyr or (D)Phe;

R^{250} is Arg;

R^{251} is (L) or (D)Leu or Lys;

R^{252} is (L) or (D)Arg;

R^{253} is (D)- or (L)- Phe;

R^{254} is Ala;

R^{255} is (D)- or (L)- Leu or is Lys;

R^{256} is absent or is (L) or (D) Arg;

R^{257} is (L) or (D) Tyr;

R^{258} is Ala; and

Y^2 is amide, thioether, thioester or disulfide.

13. The pharmaceutical composition of claim 11 wherein the IL-6 antagonist is a backbone cyclized peptide analog having the formula:

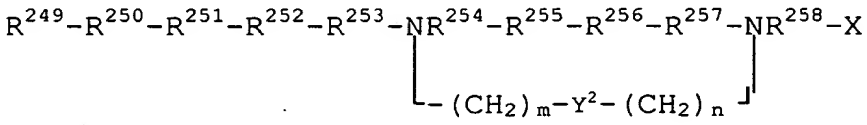
Trp-Arg-Lys-(D)Arg-Phe-AlaC3-Leu-Arg-(D)Tyr-AlaN3-NH_2

14. The pharmaceutical composition of claim 11 wherein the IL-6 antagonist is a backbone cyclized peptide analog having the formula:
(D)Lys-Arg-(D)Leu-(D)Arg-(D)Phe-AlaC3-(D)Leu-Arg-(D)Tyr-AlaN3-NH_2

15. The pharmaceutical composition of claim 11 wherein the IL-6 antagonist is a backbone cyclized peptide analog having the formula:
(D)Phe-Arg-(D)Leu-(D)Arg-(D)Phe-AlaC3-Leu-(D)Tyr-AlaN3- NH_2

16. A method for treating disorders selected from the group consisting of bacterial, parasite and viral infections, chronic autoimmune disorders and neoplasms, comprising administering to a mammal in need thereof a pharmaceutical composition comprising a therapeutically effective amount of a backbone cyclized IL-6 antagonist.

17. The method of claim 16 wherein the IL-6 antagonist is a backbone cyclized peptide analog having the general formula 1:



Formula No. 1

wherein m and n are 1 to 5;

X designates a terminal carboxy acid, amide or alcohol group;

R^{249} is Trp, (L) or (D)Lys, (L) or (D) Tyr or (D)Phe;

R^{250} is Arg;

R^{251} is (L) or (D)Leu or Lys;

R^{252} is (L) or (D)Arg;

R^{253} is (D)- or (L)- Phe;

R^{254} is Ala;

R^{255} is (D)- or (L)- Leu or is Lys;
R^{256} is absent or is (L) or (D) Arg;
R^{257} is (L) or (D) Tyr;
R^{258} is Ala; and
Y^2 is amide, thioether, thioester or disulfide.

18. The method of claim 17 wherein the IL-6 antagonist is a backbone cyclized peptide analog having the formula:
Trp-Arg-Lys-(D)Arg-Phe-AlaC3-Leu-Arg-(D)Tyr-AlaN3-NH_2

19. The method of claim 17 wherein the IL-6 antagonist is a backbone cyclized peptide analog having the formula:
(D)Lys-Arg-(D)Leu-(D)Arg-(D)Phe-AlaC3-(D)Leu-Arg-(D)Tyr-AlaN3-NH_2

20. The method of claim 17 wherein the IL-6 antagonist is a backbone cyclized peptide analog having the formula:
(D)Phe-Arg-(D)Leu-(D)Arg-(D)Phe-AlaC3-Leu-(D)Tyr-AlaN3- NH_2

21. The method for claim 16 wherein the disorder is rheumatoid arthritis.

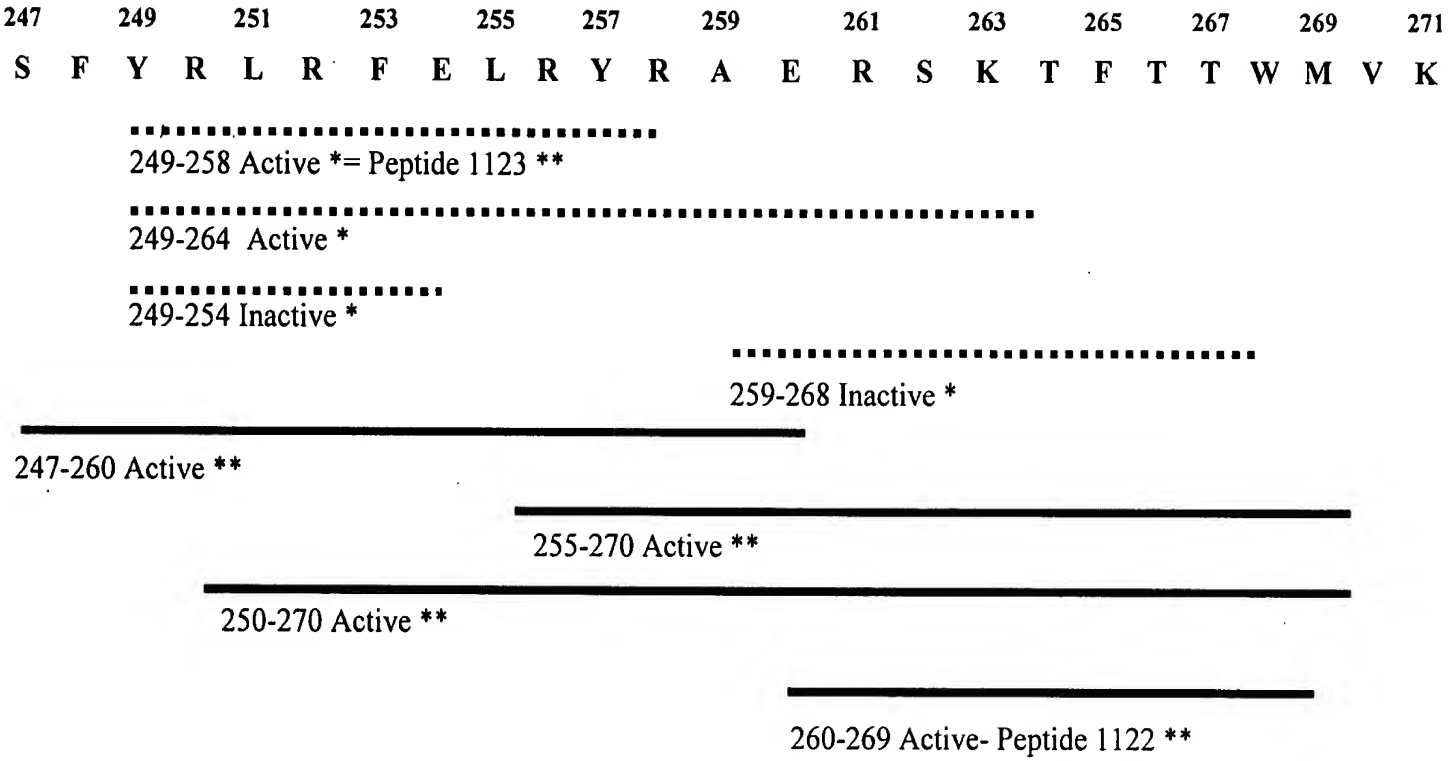
22. The method for claim 16 wherein the disorder is multiple myeloma.

CONFORMATIONALLY CONSTRAINED BACKBONE CYCLIZED IL-6 ANTAGONISTS

ABSTRACT

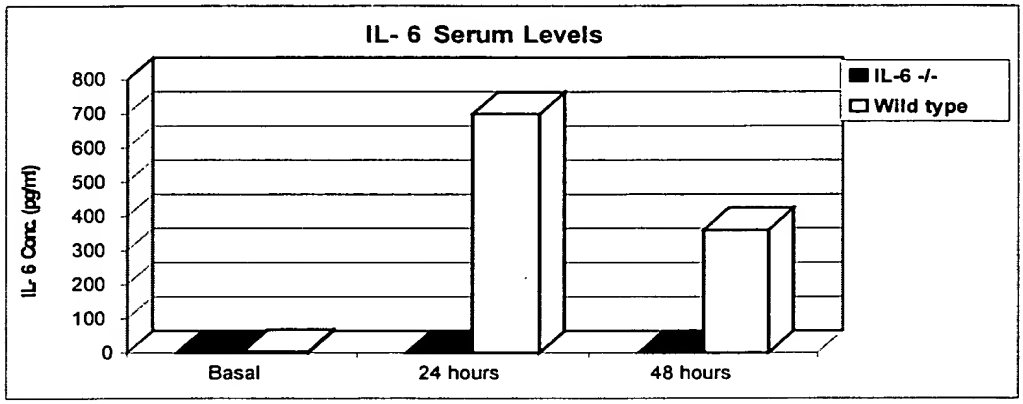
Novel peptides which are conformationally constrained backbone cyclized antagonists of IL-6, are disclosed. Methods for synthesizing the IL-6 antagonists are also disclosed. Furthermore, pharmaceutical compositions comprising IL-6 antagonists, and methods of using such compositions are disclosed.

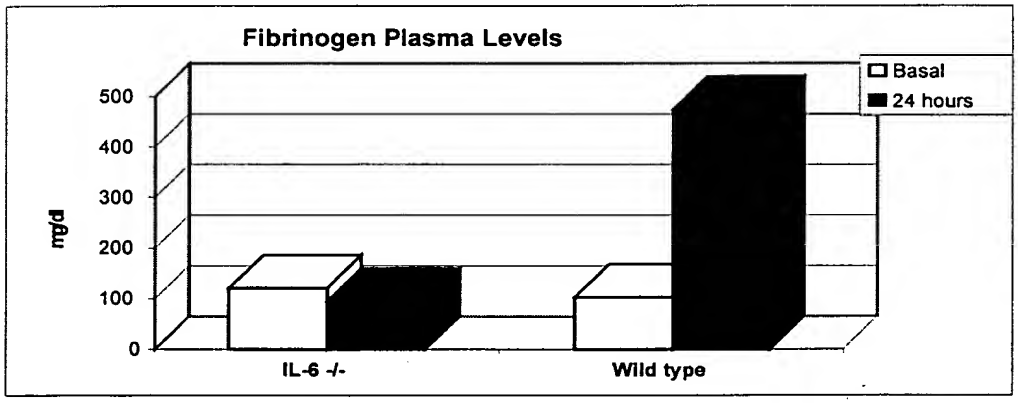
Figure 1. IL-6 inhibitory peptides derived form IL-6R sequence



* Grube and Cochran J. Biol. Chem. 269: 20791, 1994
* Halimi et al. , Eur. Cytokin. Netw. 6:135, 1995

Figures 2. The in-vivo effects of IL-6 antagonists on IL-6 mediated acute-phase responses in normal and IL-6 knockout mice:
a) IL-6 serum levels, b) fibrinogen plasma levels and c) changes in body weight.





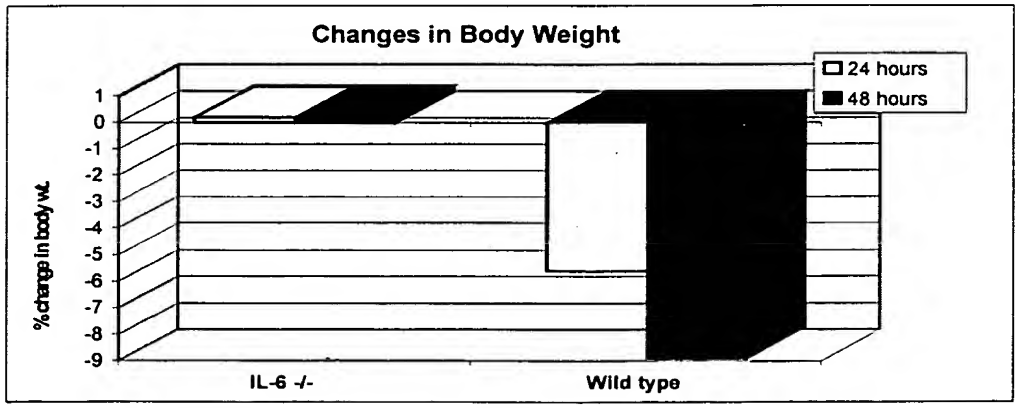


Figure 3. Restoration of Cell Growth in The Presence of Backbone Cyclic Peptide Analogs